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13. ABSTRACT (Maximum 200 Words)
The expression pattern of protein tyrosine kinase (tk) genes are often found altered in prostate cancer tissues. We developed a cDNA micro-array-based screening system to measure the expression levels of tk genes. The hardware for preparation of cDNA micro-arrays and basic protocols for hybridization were developed in year 1. In the year 2, we finished cDNA synthesis from prostate cancer cell lines and frozen tissue specimens. We optimized protocols for PCR amplification and cloning and added additional targets to our micro-arrays. Using well-characterized prostate cancer cell lines, the system delivered reproducible results during cell transformation and progression towards a more malignant phenotype. Comparing the absolute expression levels from cDNA microarrays with data from Northern blot analyses suggested that our initial approach using mixed-based oligonucleotide primers led to lowered representation of highly abundant transcripts. Furthermore, the mixed base oligonucleotide primers used in the initial cloning experiments lead to some level of unspecific amplification limiting the assay sensitivity. Problems related to primer specificity were addressed with a new primer design based on the nucleotide sequences of known genes. Furthermore, we developed a 2-step in vitro DNA amplification scheme for unbiased quantitation of tk gene expression levels in small samples.

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Table of Contents

INTRODUCTION:

Aberrant expression of receptor or cytosolic tyrosine kinase (tk) genes and, in particular, their hyper-expression are common phenomena in prostate cancer, which are believed to alter cell growth and response to external signals such as growth factors, hormones etc. Knowledge about the relative levels of expression of many tyrosine kinase genes, all at the same time, might contribute significantly to a better understanding of the processes of tumor development and progression. We are developing a rapid assay that will use innovative cDNA micro-arrays carrying small amounts of individual tyrosine kinase gene-specific targets to simultaneously determine the expression levels of up to 90 tyrosine kinase genes using a small number of cells. Four years of research and development will lead to discovery of a set of gene-specific markers associated with prostate cancer progression and a simple device capable of performing inexpensive expression profiling of these markers. The research and development efforts in the first year focused on the design and testing of robotic instruments to prepare cDNA micro-arrays carrying sets of more than 60 gene-specific tyrosine kinase fragments. The second year effort was directed towards the optimization of cDNA preparation, labeling, hybridization and detection protocols as well as the molecular cloning and sequencing of prostate cancer-specific tyrosine kinase gene transcripts. Research efforts in the third year (i.e., the reporting period 01 Mar 2002 – 28 Feb 2003) were directed towards the optimization of the in vitro DNA amplification protocols to allow tk gene expression profiling in small samples. A 12-months no cost extension will allow us to finish the proposed developments and tests including expression profiling following tissue microdissection.

During this third reporting period, Dr. Weier's effort has averaged 27%. This reduction in effort was due to Dr. Weier being on leave under the federal Family Medical Leave Act of 1993 (FMLA) since November 2001 due to his wife's disability and the birth of their daughters. In January of 2003, LBNL notified the Commander, U.S. Army Medical Research and Material Command, that the Laboratory is making a one-time extension through March 31, 2004 in accordance with Article 5, Approvals and Other Authorizations. This no-cost extension was approved by the Grants Officer, U.S. Army Medical Research Acquisition Activity on February 11, 2003 (modification #P00001 for grant number DAMD17-00-1-0085) to complete the research as proposed.

BODY:

Here, we report our progress as it relates to the approved 'Statement of Work'.

Task 3. To validate assays for multigene expression profiling in small amounts of tissues (months 18-36)

1.1 Test the system with serial dilutions of cells (months 24-30)

1.2 Test the assay with microdissected tissue from prostate cancer section (months 30-36)

We isolated total RNA from 15 different prostate cell lines (Table I) and ten frozen tissue specimens. Exponentially growing cell lines were provided to us by various collaborators, while all ten prostatic tumor tissues were obtained from the Cancer Tissue Core facility at the Comprehensive Cancer Center, University of California, San Francisco (UCSF). The actual number of processed cell lines is higher, because we received several aliquots of LNCaP cells.

Table I. Prostate specific cell lines used in our experiments.

DU145	ND-1
PC-3	LNCaP
BPH-1 CAFTD-01	BPH-1 CAFTD-02
BPH-1 CAFTD-03	BPH-1 CAFTD-04
BPH-1 CAFTD-05	BPH-1 CAFTD-06
BPH-1 CAFTD-07	BPH-1 CAFTD-08
BPH-1 TETD-A	BPH-1 TETD-B
BPH-1	

Normal prostate tissue and prostate cancer tissue specimens were obtained from the University of California Comprehensive Cancer Center (B.M. Ljung, M.D. and K. Chew). We received a total of 10 frozen tissue specimens representing 2 normal tissues and 8 cancer tissues. RNA was extracted using a commercial kit (Qiagen) and transcribed into cDNA immediately after isolation using a second kit (Ambion). Remaining RNA was stored at –80 degrees. We prepared cDNAs from the RNA by random priming and reverse transcription. Commercial kits (Qiagen, Roche, Ambion) were used for all steps. Typically, 1 μg of total RNA produced sufficient quantities of cDNA for gel electrophoretic quality control, cloning and/or repeated micro-array analyses.

Initially and mostly for the purpose of cloning expressed tk gene fragments, we performed PCR amplification for 35 cycles using about 50 ng of cDNA and mixed base primers that bind to the conserved sequences of domains VII and IX of the tyrosine kinase genes (F-TYRK: 5′-GGGCG-TACGAARRTNRSNGAYTTYGG-3′; R-TYRK: 5′-GCGCGGGCCCRWANSHCCANACRT-CNSA-3′; R=(AG), S=(TGC), N=(ACGT),Y=(CT), W=(ATG), H=(AGC)). The nucleotide sequence of these primers was based on the aminoacid sequence of the conserved domains VII and IX, and each possible nucleotide combination was included. Thus, the mixed based oligonucleotides were comprised of 1024 and 6144 different sequences for the forward and reverse primer, respectively. This high number of different sequences had two consequences: 1. The concentration of any one particular primer was very low, and 2. forward and reverse primers were used in different concentrations.

In a typical DNA amplification reaction, each PCR cycle consisted of a denaturation step of 30 sec at 94°C, primer annealing at 53°C for 60 sec and primer extension for 120 sec at 72°C. The PCR products of the expected size (~160-170 bp) were then purified by agarose gel electrophoresis. Reamplification of the PCR products has been performed with modified primers designed to include deoxy-UMP residues and a Not1 restriction site at their respective 5′-ends (F-TYRKU:5′-CUACUACUACUAGCGGCCGCAARRTNRSNGAYTTYGG-3′; R-TYRKU: 5′-CAUCAU-CAUCAUGCGGCCGCCCRWANSHCCANACRTCNSA-3′). For the purpose of cloning, the PCR products of the expected size (about 190 bp) were treated with Uracil DNA Glycosylase (Gibco/Life Technologies) and cloned into the vector pAMP1 (CLONEAMP pAMP1 System, Gibco/Life Technologies).

To prepare cDNA probes for micro-array experiments, we isolated total RNA from frozen tissues cell lines and prepared cDNAs from the RNA by random priming and reverse transcription, before we amplified tk-specific cDNA fragments with our mixed-base F-/R-TYRK primers, precipitated the PCR products and labeled them by random priming incorporating Cy3- or Cy5-dUTP.

In the course of our experiments, we discovered that this probe synthesis method can generate quality micro-array data. This method is particularly good at assaying the expression of tk genes that are poorly or moderately expressed. We subjected our results to independent validations with colleagues expert in the expression of tk genes in breast cancer cells. We have the learned that our

original method seems to recapitulate the results of other investigators (using Northern blots) for moderate and low expressing tk genes. However, we have also learned that highly expressed tk gene expression is less accurately represented in out experiments than we would prefer. The rank ordering of the high expressing genes is maintained (i.e. for tk genes that are highly expressed, a lower-expressed tk gene indeed appears to be expressed less than a higher-expressed tk genes). However, the apparent difference two highly expressed genes are less than one might expect. In essence, our original probe synthesis scheme is not completely representative of total tk expression.

We believe that the cause of this artifact is due to the depletion of degenerate primers in the early steps of PCR synthesis. Our mixed-base primer mixes contain equimolar concentrations of each the hundreds of different primers. Of course, the population of tk RNAs within and tissues sample is likely to be anything but equimolar. It is probable that the most highly expressing genes are rapidly depleting the population of degenerate primers that would be perfect matches for that species. Once perfect primers are depleted, sub-optimal primers then bind with baspair mismatches leading to degrade PCR performance.

One of our goals is to develop of assays for accurate multigene expression profiling that require only small amounts of tissue. Such an assay might ultimately allow the analysis of specimens obtained by fine needle biopsies or tissue from microdissected tumor sections. Using serial dilutions of cDNA samples to determine the assay sensitivity, we noted that cDNA isolated from about 500 cells is needed for reproducible results.

We therefore redesigned our PCR primers using the published cDNA sequences of known tk genes, and we optimized the PCR amplification scheme carefully balancing the amounts of forward and reverse primers and optimizing primer annealing conditions. Although the new primer set (F-TYRK-4, R-TYRK-4) is similar to the previous set, its complexity was decreased 1.8-fold for the new forward primer and 3-fold for the reverse primer. This lead to increased specificity and amplification efficiency. Initial measurements to test the linearity of the amplification process using artificial mixtures of tk gene fragments have shown superior (i.e., more linear) amplification results. Results shown in **Figure 1** demonstrate a better specificity of the new primers, although the overall amount of product generated seems to be somewhat reduced.

The system has been tested initially with artificial mixtures of our tk gene fragments, and then with serial dilutions of cells from prostate cancer cell lines. This allowed us to further optimize DNA amplification and hybridization parameters, and to define relevant parameters and controls. Next, we will test the multigene expression assay with microdissected sections from at least ten prostate cancer tissue specimens (in year 4). Tissues have been provided to us by Dr. Simon Hayward, Vanderbilt University Medical Center, Nashville TN, and Dr. B.-M. Ljung from the Tissue Specimen Core Facility, Cancer Center, University of California, San Francisco. Microdissection will be performed under a dissecting microscope to collect between 10 and 100 cells per sample. Surrounding normal prostate tissue and stromal cells will be used as controls to assess the detection efficiency, local variations of tk gene expression, and reproducibility of the assay. The mRNA will be isolated from the cells using commercial kits to prepare total RNA from tissues and to prepare cDNA by oligo-dT priming and reverse transcription. The RT-PCR reactions will follow the optimized protocol used to amplify tk gene fragments from cell lines. Hybridization to DNA microarrays will then allow us to determine the tk gene expression profiles in increasingly smaller numbers of cells dissected from individual tissue specimens.

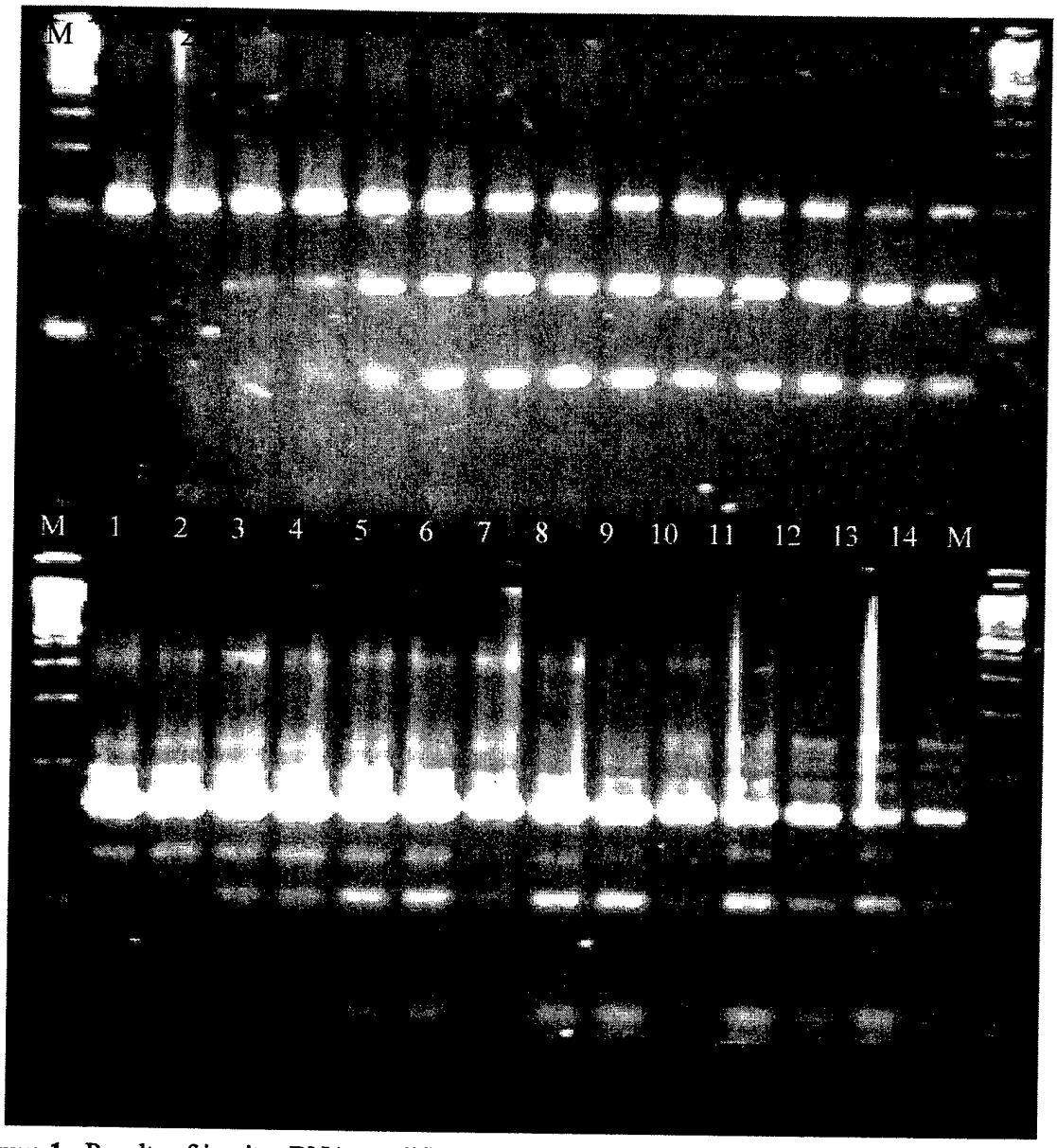

Figure 1: Results of in vitro DNA amplification using artificial mixtures of two tk fragments (PRKCD and Abl). **(A)** Reaction performed in duplicate with the newly designed primers F-TYRK-4 and R-TYRK-4 are shown on top of the gel. **(B)** Reaction performed in duplicate with the aminoacid sequence-based primers F-TYRK and R-TYRK are shown at the bottom of the gel. PCR products in A) were digested with SAU3A, which cuts the PRKCD fragments, but not the Abl-specific PCR products.

The following PCR products were loaded	**Lane**	**Sample**
	M	**100 bp size marker**
	1	**0% PRKCD/100% Abl**
	2	**0% PRKCD/100% Abl**
	3	**1% PRKCD/99% Abl**
	4	**1% PRKCD/99% Abl**
	5	**10% PRKCD/90% Abl**
	6	**10% PRKCD/90% Abl**
	7	**50% PRKCD/50% Abl**
	8	**50% PRKCD/50% Abl**
	9	**90% PRKCD/10% Abl**
	10	**90% PRKCD/10% Abl**
	11	**99% PRKCD/1% Abl**
	12	**99% PRKCD/1% Abl**
	13	**100% PRKCD/0% Abl**
	14	**100% PRKCD/0% Abl**

KEY RESEARCH ACCOMPLISHMENTS:

- Finished the isolation of RNA and preparation of cDNA from 15 prostate cancer cell lines and 10 frozen tissue specimens
- Completed the PCR-amplification of tk-specific DNA fragments and cloned the products into plasmids
- Pre-screening more than 400 cancer cell line-derived tk fragment clones and sequenced an additional 100 clones, database searches identified two clones containing potentially novel tyrosine kinase genes
- Expanded the panel of tyrosine kinase genes used for expression profiling and printed second generation cDNA micro-arrays
- Generated artificial mixtures of tk DNA fragments to be used as reference DNA
- Reconfirmed tk gene expression changes as prostate epithelial cells become tumorigenic using second generation tk micro-arrays
- Initiated search for full length cDNA clones for novel tk genes and screened BAC libraries for genomic clones
- Redesigned oligonucleotide primers to amplify more specifically thus enabling the processing of small amounts of cells or tissue

REPORTABLE OUTCOMES:

- **presentations**

2002 Annual Meeting of the Histochemical Society, Seattle, WA, July 18-21, 2002:

1. Lersch RA, Chu LW, Ito Y, Weier HUG. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level.

2. Weier HUG, Ito Y, Fung J, Lehmann L, Lersch RA, Chu LW, Zitzelsberger HF. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history.

3. Ito Y, Fung J, Hsu J, Katzir N, Lersch RA, Weier HUG. Phenotype analysis of tumor cells with eight color FISH.

4. Chu LW, Troncoso P, Johnston DA, Liang JC. Genetic alterations associated with local prostate cancer progression.

- **funding obtained**

Postdoctoral Fellowship 'Dietary Determinants of Prostate Cancer', U.S. Army Medical Research and Materiel Command DAMD 170310157, Lisa W. Chu (P.I.), 3/01/03-2/28/05

CONCLUSIONS:

This project is mostly on track and has met almost all of its milestones. The soft- and hardware components necessary for these studies were put in place in the first year. The results obtained with RNA isolated from cell lines and prostate tissues have proven the hypothesis that changes in tk gene expression can be monitored by a combination of PCR using tk gene family-specific primers and cDNA micro-arrays. While the hybridization to the DNA micro-array appears to possess the required specificity, second year research addressed the issues of hybridization background reduction and definition of a suitable reference DNA probe. Comparison of the cDNA micro-array data with those obtained by Southern blot analyses suggested a non-homogeneous amplification of tk fragments. This has been addressed by an altered PCR protocol involving new oligonucleotide primers. Due to a reduction in the P.I's effort due to a period of family medical leave, the expression profiling of small tissues samples obtained by tissue microdissection and databasing of results will be addressed in a fourth year.

REFERENCES:

None.

APPENDICES:

1. Weier H-UG, Greulich-Bode KM, Ito Y, Lersch RA, Fung J (2002) FISH in cancer diagnosis and prognostication: from cause to course of disease. Expert. Rev. Mol. Diagn. 2(2):109-119

FISH in cancer diagnosis and prognostication: from cause to course of disease

Heinz-Ulrich G Weier†, Karin M Greulich-Bode, Yuko Ito, Robert A Lersch and Jingly Fung

The last 20 years have witnessed an astounding evolution of cytogenetic approaches to cancer diagnosis and prognostication. Molecular techniques and, in particular, nonisotopically-labeled nucleic acid probes and fluorescence *in situ* hybridization (FISH)-based techniques have replaced the costly and potentially dangerous radioactive techniques used in research and the clinical detection of genetic alterations in tumor cells. Fluorescent DNA probes also enabled the screening for very subtle chromosomal changes. Clinical laboratories now choose from a growing number of FISH-based cytogenetic tests to support physician's diagnoses of the causes and the course of a disease. Depending on the specimen, state-of-the-art FISH techniques allow the localization and scoring of 10–24 different targets and overcome previous problems associated with target colocalization and detection system bandwidth. FISH-based analyses have been applied very successfully to the analysis of single cells and have demonstrated the existence of cell clones of different chromosomal make-up within human tumors. This information provides disease-specific information to the attending physician and should enable the design of patient-specific protocols for disease intervention.

Expert Rev. Mol. Diagn. 2(2), 109–119 (2002)

Most tumors are comprised of cells that escape the tightly regulated cellular life-cycle of generation, differentiation, senescence and death. Typically, genetic changes accumulate during neoplastic development and provide tumor cells with unusual growth and proliferative characteristics, most of which are inscribed in the heritable genetic code of the cells. Often referred to as mutations, these heritable changes provide genetic markers by which tumor cells might be identified and differentiated from surrounding normal cells. However, a precise determination of a normal or abnormal genetic make-up of cells often proved difficult because cells were found in different stages of the cellular growth cycle or changes were so subtle that only very few nucleic acid bases were involved.

As we learn more about the genetic changes underlying neoplastic deviation, we are also challenged to develop technologies to rapidly identify cells presenting disease-specific markers. Fluorescence *in situ* hybridization (FISH) is a nonisotopical method to visualize the location of nucleic acid sequences in cells, cell organelles, tissue sections or whole mounts of small animals. As shown schematically in FIGURE 1, FISH is based on the preparation of a nucleic acid probe labeled with a fluorescent or immunogenic hapten followed by hybridization of the probe to a DNA target rendered single-stranded by denaturation. The most simple and thus, most common way of denaturing double-stranded nucleic acids is by heating the substance above the temperature required to break the hydrogen bonds that hold together the double helix. The probe is then allowed to anneal to its complimentary nucleic acid target sequence for up to several hours and unbound probe molecules are removed by stringent washes. Most fluorochrome-labeled probes can then be seen

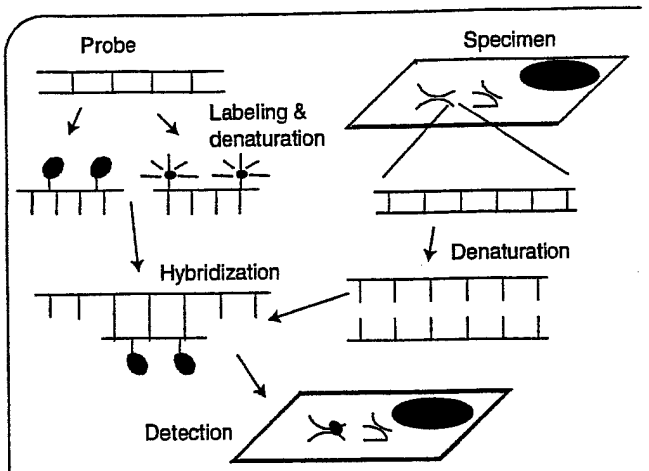

Figure 1. Schematic representation of the fluorescence *in situ* hybridization (FISH) procedure (See text for details).

directly in a fluorescence microscope. The detection of probes labeled with nonfluorescent haptens, such a biotin, digoxigenin or dinitrophenyl (DNP) typically involves the application of antibodies or affinity reagents, such as avidin or streptavidin, which carry fluorochromes for detection.

In the subsequent sections of this review, we will describe FISH applications on:

- Detection of specific numerical chromosome aberrations in tumor cells to aid diagnosis and tumor staging, to provide guidance in therapeutic decisions as well as to detect minimal residual disease
- Detection of specific structural chromosome aberrations associated with tumor development
- Genome-wide screening for numerical and structural chromosome aberrations to identify novel genes involved in the onset and progression of tumors

State-of-the-art FISH technology

Today's broad acceptance of FISH technology is a consequence of more than a decade of progress in two important areas. First, a vastly increased number of DNA probes and probes combinations are now available to aid researchers and clinicians in specific diagnostic investigations [1,2]. Second, innovative probe labeling techniques and significant advances in optical microscopy have helped to evolve FISH to the point where between 12–24 different DNA targets can be analyzed simultaneously on a routine basis [3–5]. The following paragraphs summarize the recent developments and highlight applications.

DNA repeat probes

Early applications of FISH involved the use of DNA probes that bind specifically to highly repeated DNA segments, such as the extensive arrays of satellite DNA found at the chromosomal centromeres and other heterochromatic regions [6]. The typical chromosome-specific target would be a large number of almost identical copies of the DNA repeat arranged in tandem [6,7]. Often, a single, small probe sequence is sufficient to label extensive parts of a chromosome as shown for the detection of the Y chromosomes in different species using probes of about 100–200 bp [8–10]. Strong signals are generated when the small, identical probe molecules find hundreds or even thousands of binding sites in close proximity. This also allows the detection of highly iterated tandem repeats using synthetic oligonucleotides, thus circumventing time-consuming molecular cloning, DNA preparation and labeling steps. If the probe and target are distinctly different from the rest of the genome and cross-hybridization is neglectible, probes can be applied at a vast molar excess over target sites and hybridization times may be as short as a few minutes.

Locus-specific DNA probes

The term 'locus-specific DNA probe' (LSP) refers to a single probe molecule or collection of labeled nucleic acid fragments that bind to a limited, single copy region of the genome. This typically contains a gene, a translocation breakpoint or another relevant DNA sequence. Target sizes of LSPs range from about 14 kb for lambda phage-derived probes [11] to more than a million basepairs (1 Mbp) for probes prepared from large yeast artificial chromosomes (YACs) [11–13]. LSPs have gained importance in the analysis of inter- or intrachromosomal rearrangements as well as the detection of small terminal deletions [13–16]. Access to LSPs is provided through commercial sources, such as Vysis Inc., Downers Grove, IL, USA, which offers a range of probes. These include numerous probes for known oncogenes or common translocations breakpoints. While not-for-profit organizations, such as the laboratories of Drs M Simon and J Korenberg in Los Angeles prepared extensive panels of physically mapped probes and make them available to the scientific community at minimal cost [17–20], companies, such as Research Genetics of Huntsville, AL and Children's Hospital Oakland Research Institute (CHORI, Oakland CA 94609, USA) offer a fee-for-service to screen large size insert genomic DNA libraries for clones containing a gene or sequence of interest [101–110]. As demonstrated below, multicolor FISH with LSPs allows assessment of the frequency of calls carrying specific aberrations known to be associated with tumorigenesis, analysis of the series of genetic changes that occur during tumor evolution and correlation between genotype and phenotype.

Whole chromosome painting probes

Whole chromosome painting (WCP) probes are collections of labeled nucleic acid fragments that have sequence homology with regions of the genome distributed over an entire chromosome or parts thereof. These probes are typically composed of a large number of different sequences and increasing the probe complexity, i.e., the fraction of the target that is represented by probe molecules, leads to more homogeneous staining. Different strategies including chromosome enrichment by fluorescence-activated chromosome sorting (FACS), interspersed repeat sequence (IRS) or arbitrarily-primed DNA amplification and microdissection have been applied to generate WCPs [21,22]. The main application of WCPs is the detection of trans-

locations involving nonhomologous chromosomes. WCPs are now commercially available for all human and mouse chromosomes and probes labeled with different fluorochromes can be combined to delineate as many as 24 different chromosomes. This technique, termed 'multiplex-fluorescence *in situ* hybridization' (M-FISH) [23] or spectral karyotyping (SKY) [3] will be discussed in the next chapter.

Recent advances in probe labeling & detection techniques

Labeling of nucleic acid probes has never been easier. Various companies offer kits that allow even the novice to label the DNA probes used in the planned FISH experiments. The reporter molecules of choice are fluorochromes, which if bound in sufficient quantity and density, can be observed in the fluorescence microscope without further signal amplification. In the early days of FISH, most probe DNA was labeled enzymatically by either random priming or nick translation. Today, a researcher planning to prepare his or her own probes can chose between enzymatical or chemical labeling techniques to modify the DNA [24,25].

Many projects, using such techniques as M-FISH [23] or SKY [3], require the use of relatively large amounts of multiple fluor- or hapten-labeled nucleotides for the preparation of DNA probes. Such a requirement makes these experimental approaches very expensive but the cost of such nucleotides can be reduced significantly by purchasing the chemical precursors, fluor or hapten succinimidyl esters and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (AA-dUTP) and performing simple coupling and purification reactions [26].

The way in which DNA probes are labeled has changed and the modalities of their detection have also undergone major evolution. Early probe detection systems involved nonfluorescent haptens and some kind of antibody sandwiching technique to couple fluorescent molecules to the hybridized DNA probes [21]. While these detection schemes are still widely used, novel signal amplification schemes, such as rolling circle amplification [27], have been developed to boost the fluorescence signals so that even faint signals can be detected with ease. Another very efficient technique involves the binding of an enzyme complex to the hybridized probe followed by precipitation of a fluorescent substrate [28,29]. Under normal conditions, either amplification system increases signal strength by a factor of 10–30, allowing the detection of very faint signals derived from small DNA targets [30].

An increasing number of fluorescent reporter molecules can be identified by virtue of their fluorescence emission spectrum leading to the development of a variety of multicolor schemes for the detection of numerical and structural chromosome aberrations. Some groups use optical filter-based microscope detection systems [21,31–34] for multicolor FISH to excite one fluorochrome at a time. Other laboratories [3,35,36] favor a system in which a multicolor filter or mirror provides the light for the simultaneous excitation of several fluorochromes and the emission filter is replaced by an optical interferometer (FIGURE 2). This spectral imaging system combines

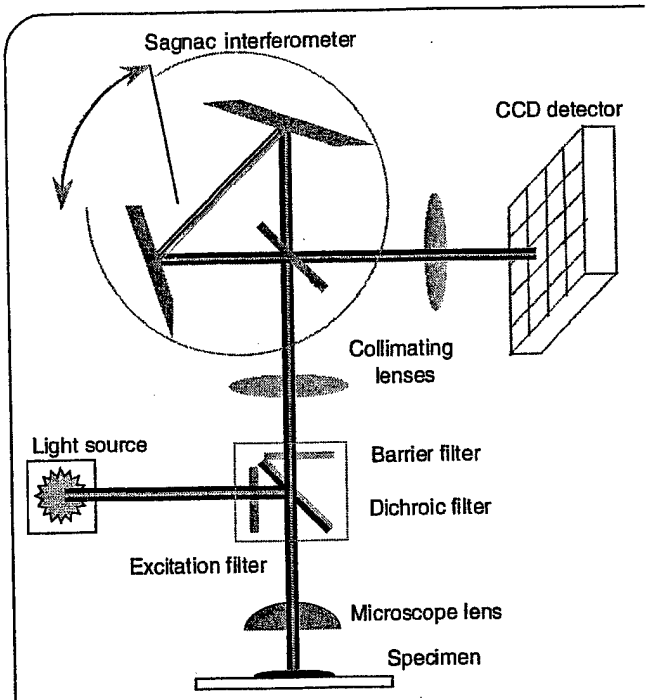

Figure 2. Schematic diagram of the optical pathway of the SpectraCube System (Image courtesy of Applied Spectral Imaging Ltd., Migdal Ha'Ermek, Israel).

the techniques of fluorescence optical microscopy, charged coupled device imaging, Fourier spectroscopy and software for digital image analysis. The power of this technology has been demonstrated by specific staining of all 24 human chromosomes in metaphase spreads, termed SKY [3,37].

The recent work of Fung *et al.* extended the application of spectral imaging to the cytogenetic analysis of interphase cell nuclei [4,35]. Five to six primary reporter molecules and a coloring scheme in which chromosome-specific probes are identified by the relative amount of each reporter molecule (termed ratio labeling) allowed scoring of up to 12 different chromosomes in a variety of interphase cell types [35]. The number of targets that can be accurately scored at the same time appears to be limited not by the labeling scheme but by the procedures available to homogeneously spread all the DNA so that spatial overlap of hybridization domains is minimized. Developed primarily for the analysis of single cells in *in vitro* fertilization and prenatal diagnosis, this technology should be easily adaptable to applications in tumor diagnosis.

Selected applications of FISH in cancer research & detection of minimal residual disease

Extra chromosomes as hallmarks of tumor cells

Trisomy 8 is observed in a significant fraction of patients with chronic myeloid leukemia (CML) [38]. In these cases, the presence of the extra chromosome 8 allows easy identification of the tumor cells and assessment of their frequency in mixed cell populations [39]. This information may become diagnostically useful and guide further therapeutic decisions. During therapy of leukemic diseases, for example, success of a specific treatment

regimen may depend on detecting early changes in the relative fraction of malignant cells in blood or bone marrow samples. FISH analysis with chromosome-specific DNA probes facilitates the differentiation between normal diploid cells and aneuploid tumor cells *via* the analysis of uncultured interphase cells. This not only reduces the time and effort required to prepare metaphase spreads for banding analysis but it also minimizes selection artifacts that might occur during cell culture [40,41]. FIGURE 3A illustrates the detection of tumor cells carrying an extra chromosome 8 among diploid bone marrow cells from a CML patient with a (47, XY, +8) tumor karyotype. Tumor cells can be identified rapidly by the presence of three hybridization signals per nucleus (FIGURE 3A, arrow).

Numerical chromosome aberrations are not limited to tumors of the hematopoietic system and they are found in a variety of solid tumors, among them renal cell cancers [42]. Metaphase spreads from these tumor cells are often difficult to obtain, thus preventing tumor studies by conventional G-banding analyses. The application of DNA repeat probes is often the method of choice for analysis of these tumors [43] and identification of tumor cells. FIGURES 3B–C show the example of interphase cells from a kidney tumor. Apparently, tumor cells are either near diploid or near tetraploid with extra copies of chromosome 8 (FIGURE 3C).

Detection of structural alterations in interphase cells

Structural chromosome alterations without gains or loss of DNA, such as reciprocal translocations, might alter the expression of oncogenes or tumor suppressor genes thus confer growth advantages to tumor cells. FISH with locus-specific probes that flank or span the breakpoint region are powerful tools to detect tumor cells as demonstrated for the bcr/abl translocation in CML [44,45]. FIGURE 3D–J illustrates the two complementary approaches. In this figure and all remaining figures, square marks next to a hybridization signal denote a red signal and triangular solid marks denote green signals. Circular open marks denote centrometic marker signals and arrows denote hybrid (red/green) signals on the same chromosome that cannot be distinguished with black and white images. Probes that span the breakpoint region can be labeled in different, i.e., chromosome-specific colors and hybridized in combination with DNA repeat probes (FIGURE 3D). As shown in (FIGURE 3F–G, cells from an individual carrying a balanced translocation t(3;4) can be analyzed for the presence and number of normal and derivative chromosomes. The probes prepared from yeast artificial chromosomes (YAC's) carry large inserts of human genomic DNA [12,13,46,47] and appear as single color hybridization domains after hybridization to normal chromosomes (FIGURE 3E and domains marked by arrowheads in FIGURE 3F). Derivative chromosomes, on the other hand, produce domains of closely spaced red and green fluorescence (arrows in FIGURE 3E,G) which can be distinguished easily from the single color domains associated with nonrearranged loci. The inclusion of one centromeric probe (marked with and open circle in this example) allows unambiguous identification of the derivative chromosomes [48]. Besides the identification of structural chromosome aberrations, hybridization of

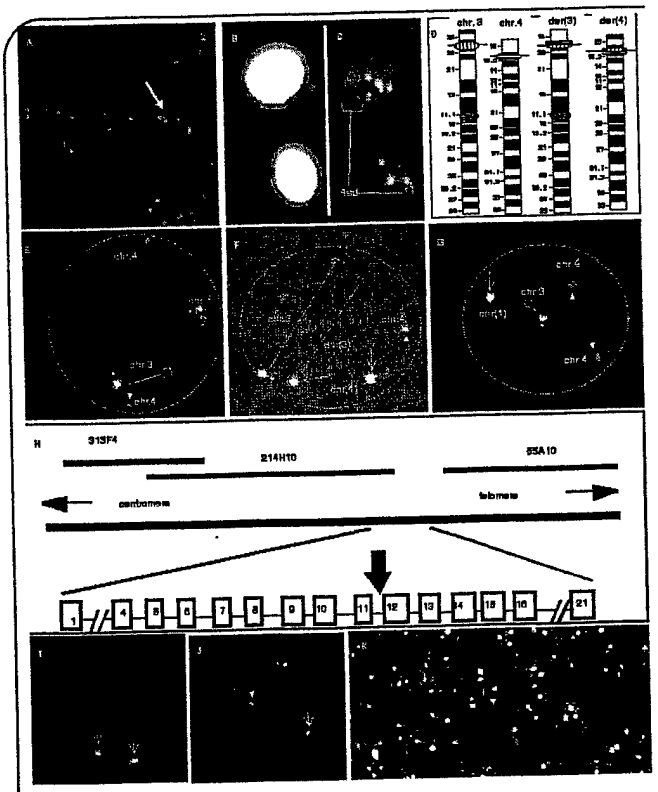

Figure 3. Locus-specific probes (LSP) detect chromosomal aberrations in interphase cells: (A) A chromosome 8–specific α satellite DNA probe identifies tumor cells in bone marrow aspirates from a leukemia patient with a tumor cell karyotype of (47,XY,+8) (arrow); **(B:** DAPI image, **C:** FISH image) In **(C)** combined hybridization of probes for chromosomes 8 (red; 5 spots indicated with lines) and 12 (green; all other spots) demonstrates the presence of cell clones of with extra copies of chromosomes 8 in interphase tumor cells in this case of a patient with kidney tumor; **(D)** A simplified hybridization scheme to detect structural and numerical chromosome alterations with probes spanning translocation breakpoints. A green probe spans a breakpoint region on chromosome 3 (oval with vertical stripes) and a probe detected in red spans the breakpoint on chromosome 4 (oval with horizontal strips). While normal chromosomes cause single color hybridization domains in interphase cells, the derivative chromosomes produce hybridization domains with adjacent red and green fluorescence (two closely adjacent ovals). Blue probes mark the centromeres of chromosome 3 thus allowing an accurate enumeration of the different chromosome types (stippled ovals); **(E)** Hybridization of locus-specific probes for chromosomes 3 and 4 to normal interphase cells produces large, single color domains representing the respective targets on chromosome 3 (green, square) and 4 (red, triangle), respectively; **(F)** Hybridization of the probes shown in **(D)** to cells derived from an individual carrying a reciprocal translocation t(3;4) shows single-color 'normal' domains (red, triangle; green, square) and the presence of translocation chromosomes in form of associated red–green domains (arrows); **(G)** Numerical as well as structural chromosome aberrations can be detected with locus-specific probes. Here, hybridization of the chromosome 3/4 probe set to embryonic cells demonstrates one copy of chromosome 3 (square), two copies of chromosome 4 (triangles) and one copy of a der(3) chromosome (arrow), i.e., aneusomy with respect to the distal parts of chromosomes 3p and 4p; **(H)** Hybridization scheme to detect ret gene rearrangements in interphase cells. Green–labeled probes bind to the 5'–end of the ret gene (313F4, 214H10), while a red labeled probe in binds to the 3'–end of the gene (55A10). The common breakpoint maps into intron 11 of the ret gene. **(I)** Hybridization of the ret–specific probes to interphase cell nuclei from normal donors reveals two–hybridization domains with associated red–green staining (arrows); **(J)** Hybridization of the ret–specific probes to interphase cell nuclei from the thyroid cancer cell line TPC–1 copy shows one red–green domain representing a nonrearranged of the ret gene (arrow) and separated green and red domains (red, triangle; green, square) indicating the rearranged copy of the second homologue; **(K)** Amplification of the c–myc gene in a case of melanoma. A touching imprint preparation was hybridized with a probe specific for c–myc (red, a center cell marked with squares at green signals), which maps to 8q24 and a chromosome 8–specific centromeric DNA repeat probe as control (Vysis CEP 8, green, a center cell marked with squares at green signals). Generally, the green signals are bright and fat; the red signals are smaller and less intense. (Image courtesy of J. Utikal, Department of Dermatology, University of Ulm, Ulm, Germany). (See online version of this article for color figures).

breakpoint spanning probes also allows the detection of numerical aberrations or aneuploidies [12,13]. In the example presented in FIGURE 3G, the reciprocal translocation in the father's germline interfered with the proper segregation of chromosomes in meiosis and led to aneuploid offspring [13,48]. In this case, hybridization involving breakpoint spanning probes as well as a chromosome enumerator (centromeric) probes helped to detect partial aneusomy in the embryonic cells [48]. The very same scheme can be applied to characterize cells from carriers of the translocation t(11;22), which is the most common translocation in humans and indicates an increased tumor risk in carriers [49].

The detection of structural rearrangements in tumor interphase cells with probes that flank one or both translocation breakpoints was first described for the above mentioned bcr/abl translocation involving genes on human chromosomes 9 and 22 [2,45]. FIGURE 3H shows a similar scheme developed to detect translocations that activate the ret proto-oncogene in papillary thyroid cancer (PTC) [50]. The common breakpoint in ret-activating translocations in PTC maps to intron 11 just upstream of the catalytic domain of this tyrosine kinase. As outlined in FIGURE 3H, we prepared probes that bind to either the 5′-end (clones 313F4 and 214H10) or the 3′-end of the ret gene (clone 55A10) and detected the bound probes with green or red fluorochromes, respectively. Mononuclear white blood cells from normal donors display the expected red/green domains (FIGURE 3I, arrows), whereas cells from the PTC cell line TPC-1 show one red/green 'normal' domain (arrow) and one set of separated red and green hybridization domains (FIGURE 3J). This was expected since TPC-1 is known to carry a rearranged copy of the ret gene [50].

Application of FISH in tumor prognostication

Numerous studies have shown that tumor development is accompanied by at least two changes:

- A change in the way cells interact with their environment *via* membrane-bound receptors
- A change in how signals originating from these receptors are transduced from the cell membrane to the cytoplam and the cell nucleus where it will alter the levels of expression of particular genes

Among the hundreds of genes involved in receptor-mediated signal transduction, only a few have been shown to be aberrantly expressed in tumors. For example, overexpression of tyrosine kinase genes, due to gene amplification or changes in the regulation of gene expression, may lead to oncogenic transformation. This has been clearly documented for the erbB-2 protein, the product of the Her-2/neu proto-oncogene and other members of the erbB family, especially in breast cancer patients. In addition, many tumors have acquired structurally altered tk proteins as well as an abnormal expression pattern through *de novo* mutational events. In cases where chromosomes have become rearranged, the catalytic domain of a tk gene can be fused to the amino terminal of another protein, thus creating a new, transforming activity as well as a new expression pattern. The above-mentioned activating ret rearrangement is just one example. Other well known examples of this mechanism of oncogene activation are the previously discussed bcr/abl-fusion protein in CML with t(9;22) and the activation of the receptor tyrosine kinase trk in papillary thyroid cancer.

Overexpression of particular genes, such as the insulin-like growth factor receptors (IGF-IRs), the epidermal growth factor receptor (EGFR or erbB) family of receptors, focal adhesion kinase (FAK) or the proto-oncogenes ret and Nyk/mer have been shown to correlate with progression to a more malignant phenotype in a variety of tumors. Other genes that have often been found amplified in solid tumors appear to be related to increased gene expression, such as the c-myc gene which maps to the long arm of human chromosome 8. Interphase FISH with locus-specific probes is able to accurately determine the number of copies of a gene per cell. Such detailed knowledge about gene amplification coupled with additional measurement of gene expression might increase our understanding of how tumors grow. Such knowledge potentially leads to the design of assays, which will allow us to perform more accurate staging of tumors and predict the course of tumor development, i.e., its capacity to grow, invade and spread to other sites.

As an example, FIGURE 3K illustrates the detection of gene amplifications in skin cancers. Tumor cells were transferred from a melanoma sample onto glass microscope slides by the touching imprint method and hybridized with a probe specific for the c-myc proto-oncogene (signals in FIGURE 3K a center cell marked with triangles at signals). A commercial probe that stains repeated DNA in the centromeric region of chromosome 8 (CEP 8, Vysis, Inc., labeled with green fluorochromes, a center cell marked with squares at green signals) was included to determine the number of whole chromosomes 8. Hybridization results (FIGURE 3K) confirm high level amplification of the c-myc gene without extra copies of chromosome 8 in these cells. This patient-specific information combined with results of population-based retrospective studies correlating c-myc gene amplification with tumor metastasis and average time of disease-free survival might allow the attending dermatologist/oncologist to prepare a more individualized therapy regimen [51].

WCP in the analysis of metaphase spreads

Whole chromosome painting (WCP) is a rapid technique to detect translocations involving nonhomologous chromosomes. High quality painting probes delineate the target chromosome from one end to the other, while the hybridization of nonspecific repeats, such as interspersed repeats or centromeric clusters of satellite DNA is blocked by addition of an excess of unlabeled repeat DNA to the hybridization mixture [22].

Simple chromosome painting experiments use only one or two WCP probes as shown in FIGURE 4A for the delineation of chromosomes 1 (square) or 8 (triangle). This is often cost-efficient and confirms a suspected rearrangement, such as the translocation t(1;9)(p36;q13) in a case of follicular lymphoma with an

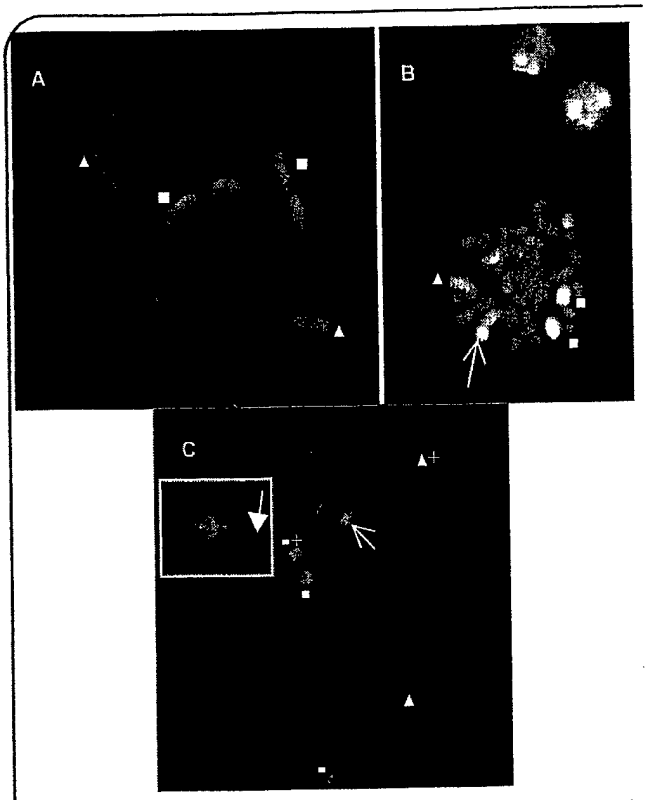

Figure 4. Whole chromosome painting (WCP) probes in the analysis of metaphase cells: (A) Dual-color hybridization delineating normal chromosomes 1 (square) and 8 (triangle) in a normal human metaphase spread; **(B)** Dual-color hybridization of WCP probes for chromosome 1 (square) and chromosome 9 (triangle) delineates normal and translocation chromosomes (arrow) in a leukemia case carrying a t(8;14) and a t(1;9). (Image courtesy of Dr. I Wlodarska, Leuven, Belgium); **(C)** Structural alterations in a thyroid cancer cell line lead to rearrangements of chromosomes 3 (square) and 17 (triangle). A dual-color hybridization reveals normal homologues (a plus mark next to the square or triangle), derivatives of either chromosomes 3 or 17 (square or triangle respectively) and a marker chromosome (solid arrowhead within insert).

additional t(14;18) shown in FIGURE 4B (the arrows point at rearranged chromosome). Using two WCP probes labeled with different reporter molecules also allows detection of translocations involving one or several stained chromosomes and any of the unstained chromosomes. FIGURE 4C illustrates this with an example from the analysis of a follicular thyroid cancer cell line. A chromosome 3-specifc WCP probe (triangle) was combined with a WCP probe for human chromosome 17 (square). An intact chromosome 3 and an intact chromosome 17 were detected. In addition, the metaphase spreads showed multiple rearrangements (open arrowhead) involving either chromosome 3 or chromosome 17 and unstained and as yet unidentified chromosomes. A marker chromosome (FIGURE 4C, insert) was found comprised of chromosome 3 and chromosome 17 material in addition to a small amount of DNA from an as yet unidentified chromosome. The unidentified material at one end of this marker chromosome was visualized by DAPI which counterstains all DNA (FIGURE 4C, solid arrowhead within insert). Chromosome painting is the method of choice to define larger breakpoint intervals in translocations like the ones shown here. Further detailed mapping of the breakpoints can then be performed by 'chromosome walking' using LSPs [12,13,46–48] or sets of probes spread along the target chromosomes in narrow distances [32,52,53].

SKY & M-FISH analyses

Complex rearrangements in tumor cells are mapped rapidly by using tumor metaphase cell preparations and combining a larger number of WCP probes. If the unambiguous classification of all chromosomes in a metaphase spread is required, the user can choose between SKY or filter-based M-FISH. The SKY approach is able to resolve fluorescence spectra with a resolution of about 10 nm, thus it records the equivalent of 30–40 distinctly different spectral images. This takes more time than recording 5–7 images with an M–FISH system and the user may want to weigh the advantages of high resolution against those of a higher throughput analysis.

FIGURE 5 illustrates the SKY analysis of a metaphase spread prepared from the human prostate cancer cell line TSUPR1. Images were acquired with an SD200 SpectraCube™ Spectral Imaging system (ASI, Inc., Carlsbad, CA). The spectral imaging system attached to a Nikon E600 microscope consisted of an optical head (Sagnac interferometer) coupled to a multi-line CCD camera (Hamamatsu). The image data were stored in a Pentium 586/300 MHz computer and analyzed by proprietary software. The multiple band pass filter set used for fluorochrome excitation was custom-designed (SKY-1, ChromaTechnology, Brattleboro, VT) to provide broad emission bands (giving a fractional spectral reading from ~ 450 nm to ~ 850 nm). Using a Xenon light source, the spectral image was generated by acquiring 80–130 interferometric frames per object. The time needed to acquire the image was less than 3 min.

The DAPI image (FIGURE 5A) helped to identify chromosomes and chromosomal breakpoint regions. Following chromosome classification based on the full 450–850nm fluorescence spectrum, individual chromosomes were assigned 'classification colors' which linked the fluorescence spectra to chromosome-specific WCP probe mixtures (FIGURE 5A) [3,37]. SKY analysis of TSUPR1 metaphases revealed a number of previously unknown translocations (t(2;8),t(3;19),t(6;7), t(6;15),t(15;18)) in the presence of several marker chromosomes (FIGURE 5D). FIGURE 5A shows the inverted image of the chromosome spread acquired through the DAPI filter and FIGURE 5B shows the WCP probe fluorescence along the same chromosomes as an RGB pseudo-color image (presented in grayscale). Based on the measurement of the complete spectrum for each point in this metaphase image, a spectral classification algorithm allowed the assignment of a defined pseudo-color to all points in the image that displayed the same spectrum. A karyotype table was obtained (FIGURE 5C) showing 84 chromosomes with six of them classified as marker chromosomes. The karyotype table (FIGURE 5D) shows the chromosomes in classification colors to the left and normal ideograms to the right. Clearly, translocations in this cell prostate cancer cell line involved the chromosomes 2, 3, 4, 5, 7, 8, 11, 15, 18 and 19.

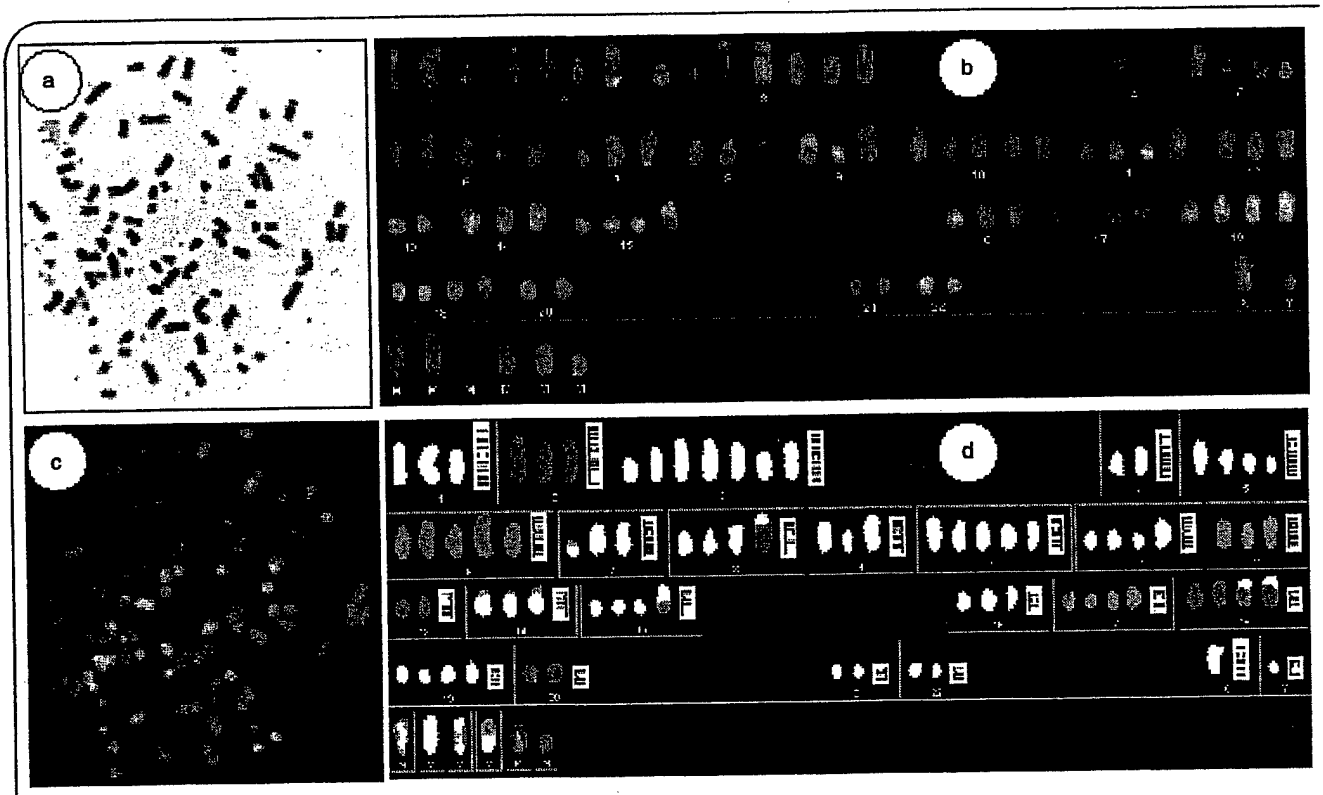

Figure 5. Spectral karyotyping (SKY) analysis of a tumor cell metaphase spread. SKY analyses of metaphase spreads from the human prostate cancer cell line TSUPR1: **(a)** Inverted DAPI image; **(b)** RGB pseudo-color image rendered in grayscale; **(c)** Karyotype table in grayscale; **(d)** Spectral karyotype showing chromosomes after assignment of classification colors to the left and normal idiograms to the right rendered in grayscale. Figures reproduced with kind permission of Springer Verlag [35].

The application of M-FISH is illustrated by the example shown in FIGURE 6. Metaphase spreads from a primary culture of cells from a female acral lentiginous melanoma (ALM) patient were hybridized with SpectraVysion probe mixture (Vysis) and analyzed using an M-FISH microscope system. The M-FISH images reveal several numerical and structural abnormalities summarized as karyotype of 44,X,+7,-10,-X,del(2q),t(1;8),t(3;22),t(6;15), t(3;22), del (2q).

Both techniques, SKY as well as M-FISH, have detection limits in the megabasepair range [54]. The most common problem with the detection of small chromosomal fragments in complex translocations is the spatial overlap of combinatorially labeled probes. As Lee *et al.* [54] pointed out, the juxtaposition of material from nonhomologous chromosomes frequently results in overlapping fluorescence at the interface of the translocated segments, a phenomenon also referred to as 'flaring' [55]. In most cases, the negative impact of flaring and chromosome misclassification can be addressed by subsequent hybridization using chromosome-specific WCP probes [54].

Conclusion & expert opinion

Intensive biomedical research in the last two decades has increased our understanding of genetic changes underlying tumorigenesis and progression to a more malignant tumor phenotype. The International Human Genome Project and its many associated efforts have generated resources and tools that now enable researchers to identify genetic changes at the level of single cells.

Playing a pivotal role in the genetic analysis of single cells, FISH has frequently helped to unravel the complex changes accompanying tumor invasion and metastasis. FISH owes its wide acceptance to the fact that it has become a simple procedure bringing together two main components: cells and tissue specimens (provided by the researcher or clinician) and nonisotopically-labeled probes, many of which are now commercially available. The near future is likely to see a significant increase in the number of probes cleared by the US FDA for *in vitro* diagnostic use as well as ready-to-use reagents pushing the limits of detection to smaller targets and, at the same time, increase the number of targets that can be studied simultaneously. Instrument prices are unlikely to change but increased automation and knowledge-based information systems will facilitate the molecular cytogenetic analyses and reduce costs.

The FISH-based assays will be rapid, inexpensive and require only a small number of cells, thus providing an affordable diagnostic service to the large community of cancer patients in the USA and elsewhere. Information gained by these measurements can be utilized to enhance prognostication, therapeutic decisions and patient management or to measure the effects of drugs in laboratory as well as clinical studies. We steadily increase our knowledge about the relationship between genetic alterations and the course of the disease. Determining genetic changes beside the status of biochemical and histopathological markers will enable clinicians to provide patients with a more individualized therapy.

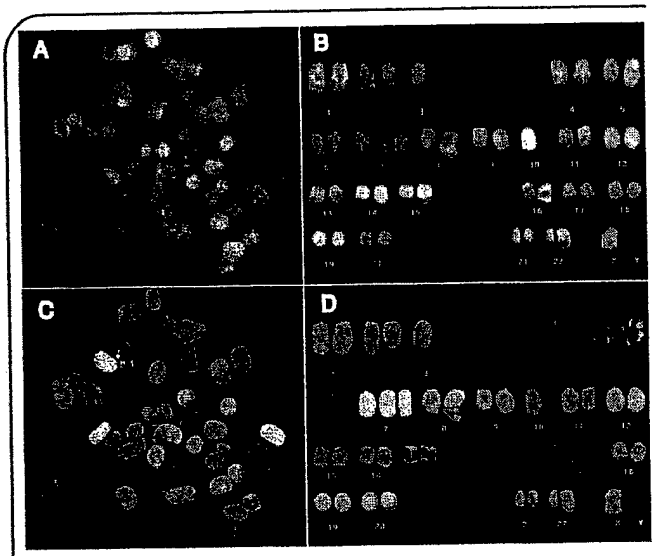

Figure 6. M-FISH analysis of human metaphase spreads reveals structural alterations in a case of acral lentiginous melanoma (ALM). Metaphase spreads from a primary culture of cells from a female ALM patient were hybridized with SpectraVysion probe mixture (Vysis, Inc.) and analyzed using an M-FISH microscope system. The M-FISH images reveal several abnormalities summarized as karyotype of 44,X,+7,-10,-X,del(2q),t(1;8),t(3;22),t(6;15), t(3;22), del (2q). **(A)** RGB colored image of the metaphase spread rendered in grayscale; **(B)** the karyotype table in in grayscale; **(C)** the metaphase image after assignment of classification colors rendered in grayscale; **(D)** the karyotype table in classification colors rendered in grayscale

Five-year view

Rapid progress of the Human Genome Project and the completion of a draft sequence of the human genome have provided a course for even more comprehensive genetic analyses in the future. As key events and changes during the progression of normal cells to malignant tumors are deciphered, FISH probes and hybridization protocols will be developed to visualize these changes in individual cells. To the clinician, this will provide important information complementing histopathological analyses and thus may guide individualized therapeutic decisions.

While multicolor FISH techniques are poised to help decipher the complex changes underlying cancer, the early detection of tumor cells or minimal residual disease as well as identification of individuals at elevated risk faces several challenges. First, FISH technology must go beyond a simple detection of the somewhat static cytogenetic changes and address the relative levels of expression of genes involved in the tumor phenotype. A typical example is the overexpression of the erbB-2 protein, the product of the Her-2/neu proto-oncogene, in various forms of cancer. There is growing evidence that gene amplification in addition to overexpression is an important prognostic factor in breast cancer and future FISH techniques should be able to render information about the levels of both, amplification as well as gene expression.

Other concerns are directed towards the amount of material needed to perform a comprehensive genetic analysis of tumor cells by FISH. The successful analysis of single blastomeres biopsied from preimplantation human embryos [47,48,56] has opened up new avenues for the analysis of small samples. Future technology developments will be geared towards the generation of a large amount of cytogenetic information from a small number of cells.

As the main thrust of research in the postgenome era shifts to proteomics, FISH techniques will need to change and accommodate immunocytochemical techniques, or minimally, be able to combine nucleic acid analyses with protein quantitation and phenotypic characterization using tumor marker-specific antibodies. Second, to conduct population studies, FISH technology needs to become standardized and automated. Present hands-on procedures must be modified to allow the processing of hundreds or even thousands of specimens within reasonable timeframes. This also calls for knowledge-based, automated analysis systems able to gather and categorize information at very high speed. While most of the necessary technology already exists, it will take continued funding and engineering ingenuity to bring the pieces together. Finally, the power of FISH-based techniques for patient diagnosis is now beginning to be harnessed. Many health professionals outside the field of molecular genetics must be trained to correctly interpret FISH data. Computer professionals, who will design and implement future software and primary care professionals, who work directly with the patients, must be trained to understand FISH data. This training may turn out to be as challenging to achieve as the technical horizons we have mapped out above.

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Key issues

- FISH is non-isotopic method to localize nucleic acids, such as DNA and RNA.
- Increased application of FISH assays in the clinical practice will require thorough training of healthcare professionals.
- Probes used for FISH are labeled with nonradioactive haptens, such as biotin, digoxigenin or fluorochromes. They are stable for several years and can be disposed of without hazards. Several FISH probes labeled with different reporter molecules can be combined for multilocus analyses. Many FISH probes are now commercially available.
- FISH assays can be set up to investigate specific alterations which are typical for a particular tumor or they can be designed to screen the entire genome for alterations.
- FISH is sensitive and rapid.
- FISH results support the molecular staging of tumors, thus facilitate prognostication and individualized treatment regimens.
- FISH assays detect numerical as well as structural alterations in a wide variety of cells and tissues and, as our understanding of the cytogenetic changes underlying tumorigenesis, invasion and metastasis increases, will evolve to specifically address the etiology of each disease.
- To be able to handle a significant larger number of clinical samples, FISH techniques need to be automated. There is also a growing need to design digital imaging systems and expert computer systems for FISH analyses in the clinical laboratory.

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